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14. ABSTRACT

We have developed an electrophoretic mobility shift assay for assembly of the core ligase complex involved in nonhomologous end joining (NHEJ). The assay can detect inhibition of complex formation upon linkage to estrogen receptor. We have demonstrated that Cernunnos is an excellent target for disruption of NHEJ. We have shown that B. subtilis Sfp enzyme can attach biotin-CoA and TAMRA-CoA conjugates to ybbR-tagged Cernunnos without disrupting its ability to stimulate end joining. We synthesized hydroxytamoxifen-CoA as a prototype adapter for attachment to ybbR-tagged Cernunnos. This will allow us to demonstrate that end joining is preserved in the absence of estrogen receptor, and disrupted in its presence.

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DNA repair, molecular adapter, nonhomologous end joining

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Project title: Targeting breast cancer with a steroid adapter to inhibit DNA repair

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Introduction

Targeted therapy offers the hope of curing cancer without side effects. We propose to synthesize a molecular adapter that will chemically link a DNA repair protein to the estrogen receptor (ER). The adapter will consist of hydroxytamoxifen attached to a small molecule warhead that binds a protein involved in the repair of DNA double-strand breaks. Binding of the adapter to the repair protein will not affect DNA repair. However, in cells expressing ER, the hydroxytamoxifen component of the adapter will bind ER and the warhead will bind the DNA repair protein to create a complex that disrupts assembly of the complex and/or misdirects the repair protein away from double-strand breaks to ER binding sites in the genome. The adapter will be administered together with agents that generate DNA double-strand breaks, such as ionizing radiation or doxyrubicin, which are already highly effective treatments for breast cancer. *The molecular adapter will make cells expressing ER hypersensitive to already effective treatments, while sparing tissues that do not express ER.*

Body

Task 1: Conduct feasibility studies for targeting XL

Subtask 1.1. Determine which NHEJ protein would be the best target for the molecular adapter.

Potential targets for disrupting the NHEJ pathway include XRCC4/Ligase IV (XL) and Cernunnos (C) (Fig. 1). The figure shows a molecular adapter linking Cernunnos to the estrogen receptor. XL is also a potential target because of its central role in NHEJ. Both Cernunnos and XL are excellent targets because of their low abundance in cells. We chose to focus on Cernunnos for three reasons. The cellular abundance of Cernunnos even lower than that of XL (Ahnesorg et al., 2006). Second, an electrophoretic mobility shift assay (EMSA) showed that multiple molecules of Cernunnos assemble into a NHEJ repair complex on DNA ends, thus providing more targets for the molecular adapter (Fig. 2). Third, we can purify large quantities of Cernunnos protein in a bacterial expression vector (whereas XL requires purification from a more cumbersome baculovirus system). Thus, we have focused on Cernunnos as a target for the molecular adapter.

Previously, we showed that Cernunnos stimulates the joining of blunt or mismatched ends in vitro (Tsai et al., 2007). In our strategy, the molecular adapter will interfere with NHEJ in the presence of ER protein by one of two mechanisms: direct interference with assembly of the MEnd ligase complex on DNA ends, or mis-localization of a key NHEJ protein to binding sites for the ER (Fig. 1).

This subtask has been completed.

Subtask 1.2. Show that modification of XL to His-XL preserves its activity in NHEJ.

Previously, we purified modified XL (His-XL) containing 6 histidine residues at the C-terminus of Ligase IV. His-XL together with purified Ku and Cernunnos reproduced MEnd ligase activity. This demonstrated that we could modify the C-terminus of Ligase IV without affecting MEnd ligase activity. This subtask has been completed.

Fig. 1. Strategy for disrupting NHEJ in cells expressing estrogen receptor.

The normal NHEJ pathway (left side of the diagram) requires Ku, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XL, and Cernunnos (C). DNA-PKcs facilitates synapsis of the DNA ends (DeFazio et al., 2002). Ku, XL and Cernunnos join even DNA with mismatched ends, which we term MEnd ligase activity (Tsai et al., 2007). Polymerase and nuclease process the DNA to permit ligation of both DNA strands.

As depicted in the legend (upper right corner), the molecular adapter consists of hydroxytamoxifen (black) covalently linked to a small molecule (red) that binds Cernunnos. The adapter preserves NHEJ in the absence of ER, but disrupts NHEJ in the presence of ER due either to mislocalization to ER binding sites or to interference with Cernunnos' interactions with other NHEJ proteins.

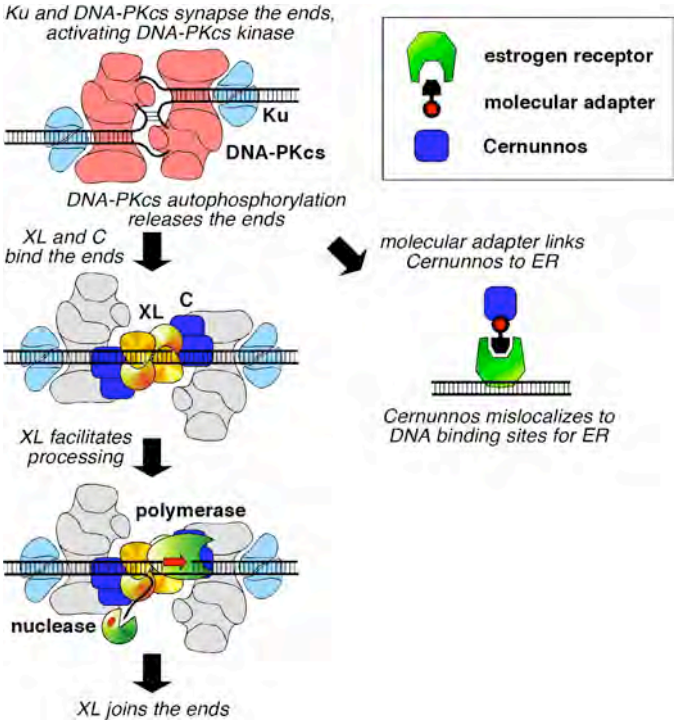
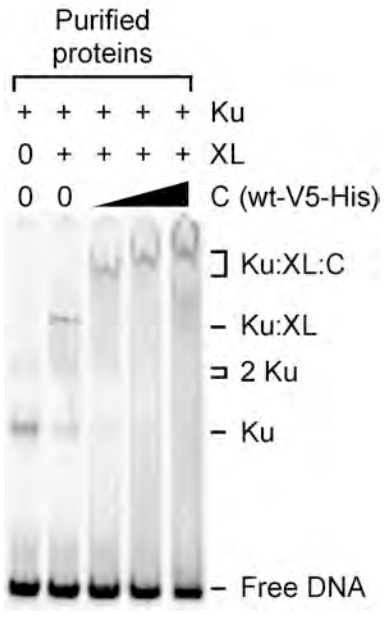


Fig. 2. EMSA for MEnd ligase complex on DNA

DNA was incubated with purified proteins: Ku, 2.4 nM; XL, 9.6 nM; and Cernunnos (C), 4.8 nM, 9.6 nM and 19.2 nM. Non-denaturing gel electrophoresis resolved complexes of DNA bound by Ku (lane 2), Ku and XL (lane 3) and Ku, XL, and Cernunnos (lanes 4-6). Free DNA migrated to a position near the bottom of the gel. Ku:XL:C denotes the position of the MEnd ligase complex. Increasing concentrations of Cernunnos generate progressively larger MEnd ligase complexes on DNA, suggesting cooperative binding of up to 3 molecules of Cernunnos to the protein-DNA complex. To emphasize the shift in mobility, Cernunnos was modified with a V5-His tag at the C-terminus. The modification preserved the MEnd ligase activity of the complex (C. Tsai and G. Chu, unpublished data).



Modified Subtask 1.3. Use ybbR-Cernunnos to test our cell free systems on a prototype adapter.

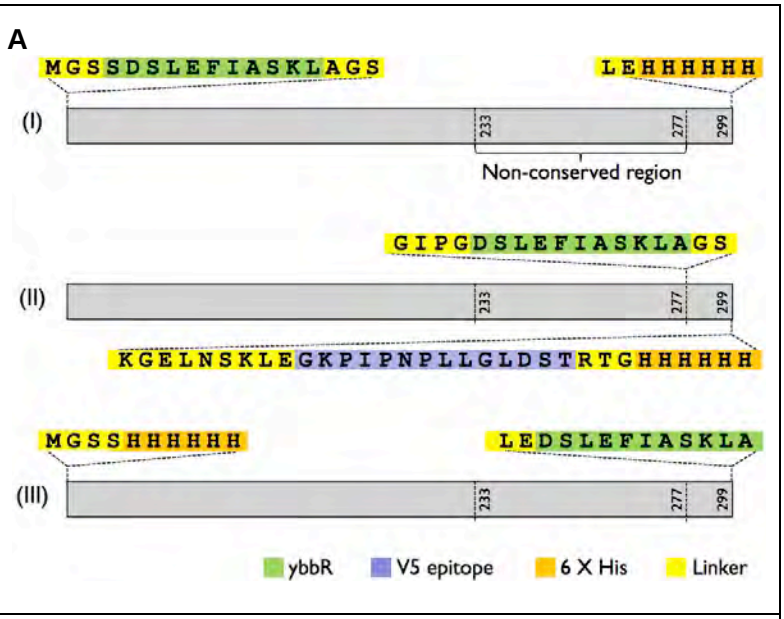
To construct a prototype adapter, we are now using a ybbR tag, which is an 11 amino acid substrate for B. subtilis Sfp phosphopantetheinyl transferase. Sfp attaches CoA attached to small molecules to the ybbR substrate (Yin et al., 2005). We constructed recombinant Cernunnos with a ybbR tag at the N-terminus, C terminus and near the C-terminal end of a long non-conserved region in Cernunnos (Fig. 3).

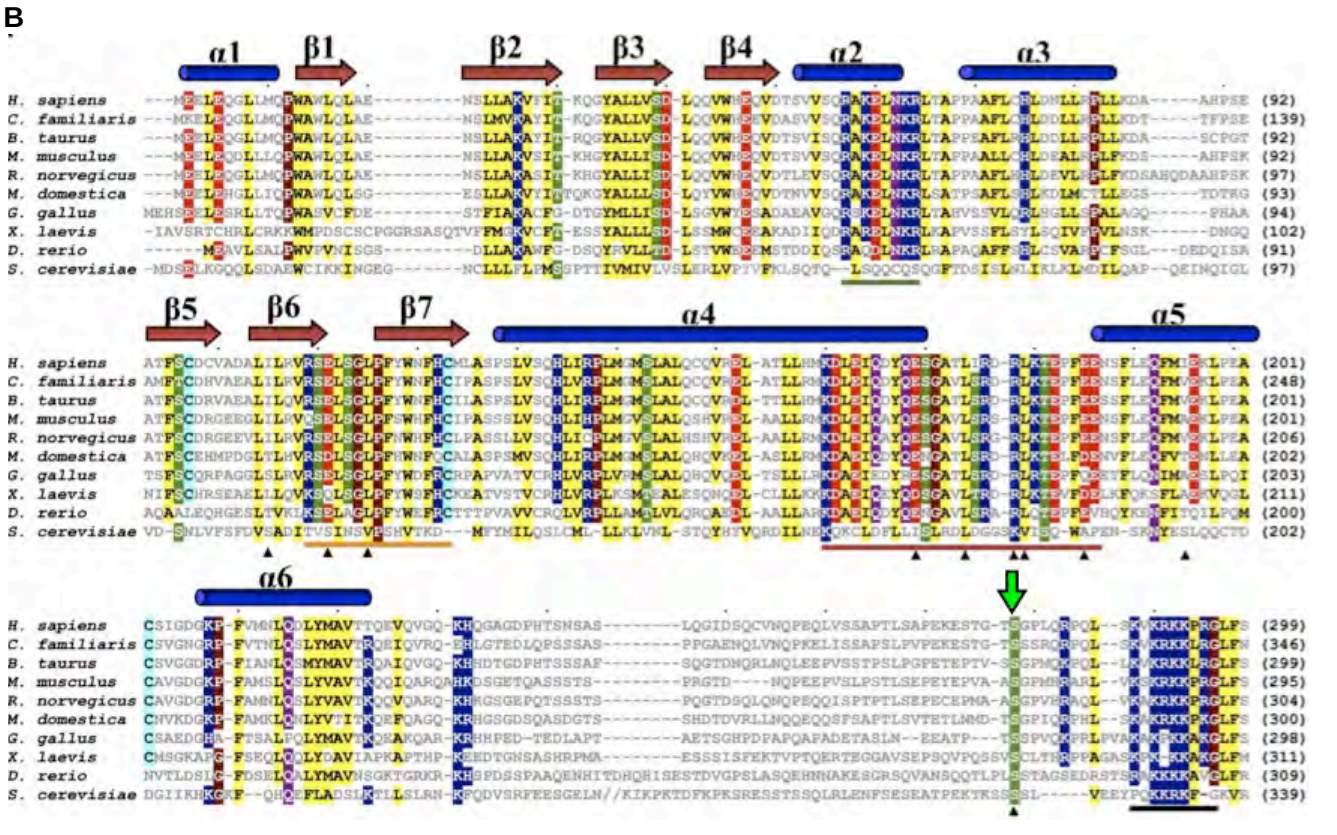
We previously reported the expression and purification of biochemically active Cernunnos that had been modified with the ybbR tag at the N-terminus (construct I in Fig. 3). We used Sfp and biotin-

CoA to label the ybbR tag with biotin, and purified the biotinylated protein on a streptavidin mutein (mutated streptavidin) matrix.

The N-terminal biotinylated Cernunnos molecule remained biochemically active. Attachment of streptavidin to biotinylated Cernunnos preserved MEnd ligase activity. Attachment of streptavidin (a 53 kDa tetramer) mimicked attachment of the estrogen receptor (a 134 kDa dimer) to Cernunnos. Thus, the N-terminus presents a potential target site for the molecular adapter, in which inhibition involves mislocalization of Cernunnos to ER binding sites.

Fig. 3. Cernunnos with ybbR tags
A. Cernunnos constructs. Cernunnos contains a non-conserved region from amino acid 233 through 277. We constructed three recombinant proteins: (I) a ybbR tag with a 2 amino acid linker is fused to the Cernunnos N-terminus; (II) a ybbR tag with linkers is inserted just after T277; (III) a ybbR tag is fused to the C-terminus. V5 epitope and His tag were inserted as indicated.
B. Cernunnos amino acid sequence. Conserved amino acids have colored backgrounds. Blue and red bars indicate α-helical and β-sheet structures. The green arrow marks the insertion site for the ybbR tag in Construct II.





We modified Cernunnos with the ybbR tag at the C-terminus (construct III in Fig. 3). However, this construct failed to support robust expression, and we did not study it further.

Next, we inserted the ybbR tag at an internal site of Cernunnos, the C-terminal end of the non-conserved region (construct II in Fig. 3). We are currently testing whether this construct supports robust expression. The insertion site for ybbR is close to the conserved basic region near the extreme C terminus, which we previously showed was required for DNA binding and NHEJ activity. We have now purified this recombinant Cernunnos molecule to near homogeneity and will test it for MEnd ligase activity once we have achieved homogeneity.

Fig. 4. Synthesis of the hydroxytamoxifen-CoA prototype molecule

The top line shows the starting compounds propiophenone, Compound 1, and 4,4'-hydroxybenzophenone, Compound 2 (both from VWR International). We synthesized the diphenol following the protocol of (Yu and Forman, 2003). We then modified the diphenol by addition of an azide group to yield Compound 3, following a modification of the protocol of (Trebley et al., 2006). The figure depicts Compound 3 in the Z (zusammen)-form, whereas the reaction utilizes either of the two hydroxyl groups to generate equal amounts of Z-form and E (entgegen)-form. Only the Z-form binds to ER, but this is not a problem, since Z and E-forms interconvert readily at room temperature.

The middle line shows conversion of CoA to an alkyne, Compound 4. The bottom line shows tamoxifen, hydroxytamoxifen and the final product, hydroxytamoxifen-CoA, which was formed by joining the Compound 3 azide and the Compound 4 alkyne via the “click reaction”, an azide-alkyne Huisgen cycloaddition (Kolb et al., 2001). Successful synthesis of each intermediate and the final product were verified by mass spectrometry (see Fig. 5). Conversion of input CoA to the final product occurred with 35% efficiency, yielding 3.5 mg of hydroxytamoxifen-CoA.

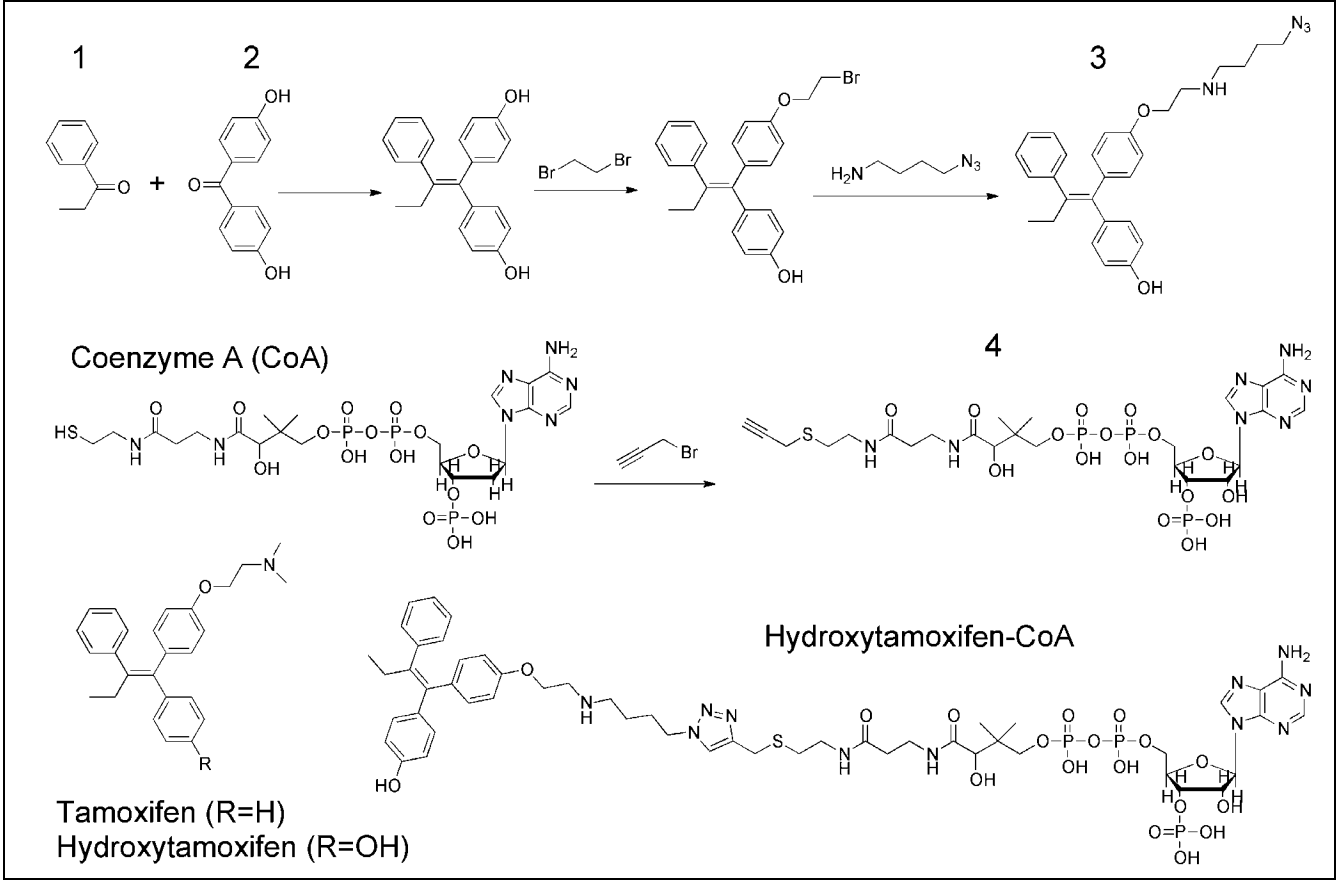
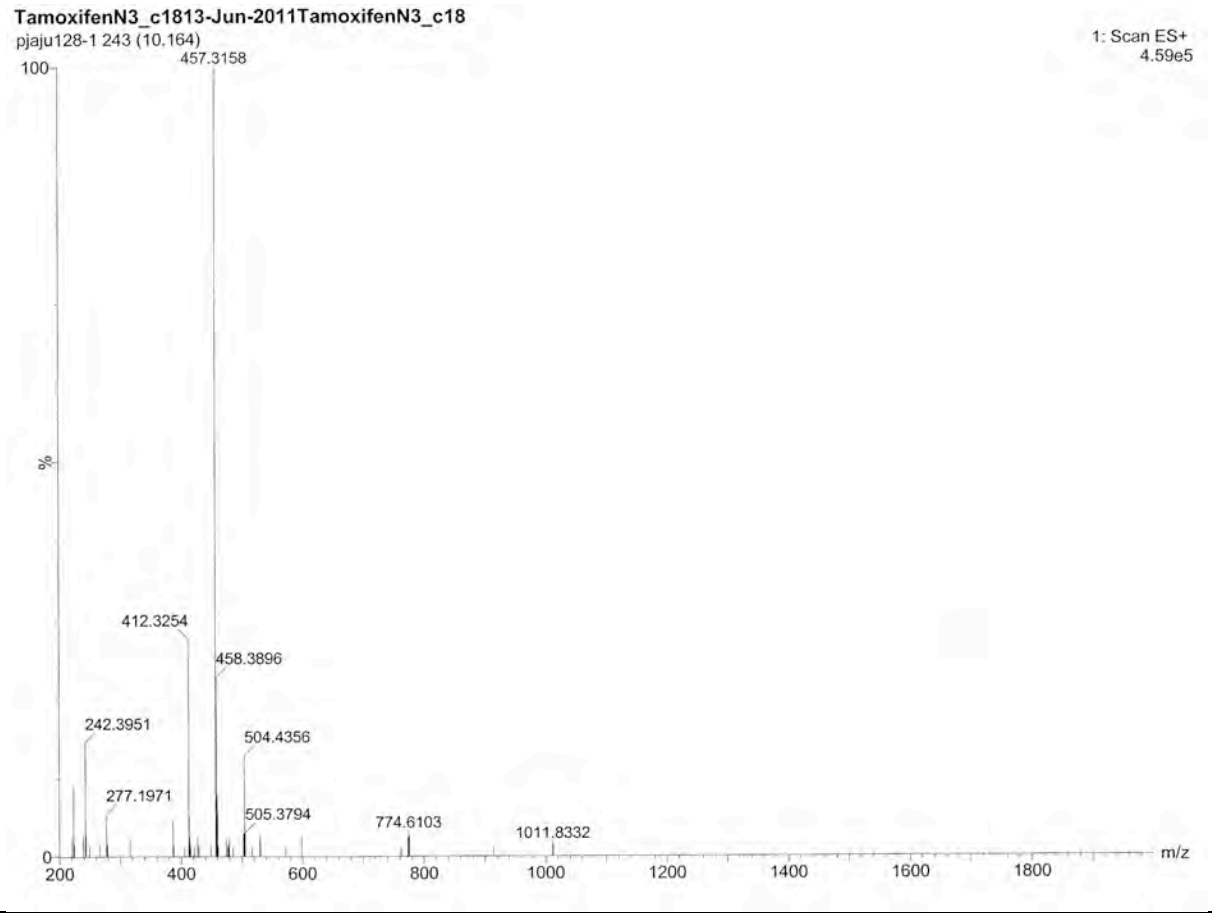


Fig. 5. Mass spectroscopy profile from synthesis of the hydroxytamoxifen-CoA prototype molecule

The representative mass spectroscopy profile shows the product of the multi-step synthesis of Compound 3 (see Fig. 4). The remaining reactions are simpler single step syntheses. The predicted molecular mass of Compound 3 is 456.25. The mass spectroscopy peak of 457.3158 is shifted by the mass of a proton from ionization of Compound 3.



After modifying the protocol of Trebley et al., we successfully attached hydroxytamoxifen to CoA (Trebley et al., 2006). We used mass spectroscopy to verify successful synthesis of the intermediates and the final product. We are preparing to use Sfp to attach the hydroxytamoxifen-CoA conjugate to Cernunnos tagged with ybbR at the N terminus.

In pilot experiments, we have successfully attached Cernunnos to biotin-CoA (as described above) and to the fluorescent dye TAMRA-CoA. Both reagents were used because they permitted rapid assays to measure successful attachment.

Based on our results with Cernunnos attached to streptavidin at the N-terminus, we expect that addition of ERα (Invitrogen) will fail to disrupt the activity of Cernunnos attached to hydroxytamoxifen at the N-terminus. However, we would expect that addition of circular DNA containing ER binding sites will mislocalize the Cernunnos and prevent assembly of the MEnd ligase complex.

Task 2: Select for small molecules that bind to Cernunnos or XL

Our collaborator, Pehr Harbury, has constructed a combinatorial tri-peptoid library on DNA with 2.2×10^{10} members. The library is constructed in 4 synthetic steps with final molecular weights averaging near 500 Daltons. This new scheme increases diversity of the scaffold, and allows other

sources of building blocks. We will use the new library to select for small molecules that bind to Cernunnos or XL.

Task 3: Synthesize an adapter for the estrogen receptor that inhibits DNA repair.

Our progress in Task 1 allows us to test prototype adapters for the estrogen receptor utilizing hydroxytamoxifen, which has a very high binding affinity for the ER, and is the most potent form of tamoxifen. Furthermore, an adapter containing hydroxytamoxifen will have less potential for stimulating the growth of breast cancer cells upon binding to the ER.

Key Research Accomplishments

We have developed a reliable protocol for synthesizing hydroxytamoxifen-CoA. This prototype adapter molecule will be tested for its ability to inhibit MEnd ligase activity only upon addition of ERα. Successful synthesis of the prototype adapter means that we can use the same chemistry for synthesis of the peptoid-CoA adapter.

Reportable Outcomes

1. We will prepare a manuscript describing assembly of the MEnd ligase complex. That manuscript will include the EMSA reported here.
2. This grant has supported the employment of Dr. Chun Tsai as a research associate and Ms. Prajakta Jaju as a research assistant.

Conclusion

We have developed an EMSA capable of detecting assembly of the MEnd ligase complex. The EMSA can also detect inhibition of complex formation upon linkage to a large protein such as ER, either by steric interference or by mis-localization to another DNA binding site.

We have demonstrated the feasibility of using the ybbR tag to screen regions of Cernunnos that can serve as targets for disruption of NHEJ.

We have successfully synthesized hydroxytamoxifen-CoA as a prototype adapter to be used as proof-of-principle for our strategy of targeting breast cancer. We will use Sfp enzyme to attach hydroxytamoxifen to Cernunnos, and test whether ERα disrupts MEnd ligase activity.

After conducting these experiments, we will screen the Harbury Lab peptoid library for small molecules that bind to the non-conserved region of Cernunnos.

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